点や，血液の所見などから否定的と思われました。薬剤性の好酸球性肺炎については発症当時使用していた薬剤についてDLSTを施行しましたが陰性の結果を得ており，薬剤性の要素も否定的です。PIE症候群とCMV感染の関連性については，肺生検組織中のCMVのDNAに関しては陰性であり，病理組織所見で巨細胞を認めない点から懐疑的ですが，否定する根拠は得られていません[1)2)]。

最後に退院後に発症した側頭動脈炎についてですが，こちらは特徴的な臨床所見から外来主治医が精査を推めて確定診断されました。病理所見では巨細胞の存在が確認できませんでしたが，側頭動脈炎に矛盾しない所見でした。本症例では側頭動脈生検組織からCMVのDNAは検出されず，両者の関連性については不明ですが，海外の文献では側頭動脈炎の生検組織中に有意にCMVのDNAが検出されたとの報告もあり，PIE症候群の場合と同様に完全に否定する証拠は得られていないと思われます[3]。

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〔質疑応答〕

座長（三崎）　それまで健康だった40歳の男性例で起こった，興味深いエピソードと経過の報告です。ご討議お願い致します。

金子（順天堂大学医学部附属順天堂浦安病院内科）　確認させていただきたいのは，病初期でサイトメガロウイルスのIgM抗体陽性，C7-HRP陽性より，要するにウイルス血症が起きたという判断でγ-globulinだけの治療を行ったのか，ガンシクロビルを含めた抗ウイルス療法を行ったのかを聞かせてくださ

い。

演者　この時点では，ガンシクロビル等は使用しておりません。その理由ですが，他のウイルス感染等の細菌感染がすべて否定されていなかったということで，まずγ-globulinから使用開始しました。

金子　経過中，CD4陽性T細胞数などをおわれましたか。

演者　1回行っていたと思いますが，顕著な低下はなかったと思います。

座長　経過表では少し分かりにくかったのですが，末梢の好酸球数が肺の症状が出るとともに好酸球が増多しているようにお見受けしました。何パーセント，あるいは何個ぐらいまで増えたのか。あるいは，次に側頭動脈炎の症状が出たときにも，やはり好酸球の増多があったのかについてお伺いしたいのですが。

演者　PIEが出現する病初期は，WBC 1万2000～3000のうち，70～80%ぐらいで好酸球が見られました。側頭動脈炎を発症した当時は，eosinoに関しては上昇を認めておりません。

座長　ANCAはいかがですか。

演者　ANCAは，p-ANCA，c-ANCAとも陰性でした。

座長　DLST陰性ということですが，PIE様の肺病変が出たとき，薬は何を使っていらっしゃいましたか。

演者　H_2ブロッカー，粘膜保護剤と，抗生剤でサーシリンが入っていたのですが，既に好酸球が上がってきた後に使い始めたものですから，関連性は薄いと思っています。

座長　DLSTで陽性が出ることのほうが逆に少ないと思いますので，DLST陰性だけを根拠に「否定的」と言い切るのは，少し難しいかと思います。

演者　そうですね。また，ステロイドパルス直後の血液で行っていますので，先生おっしゃるように断定はできないと思います。

越智（東京医科歯科大学生体応答調節学）　まず1点ですが，側頭動脈炎やPIE症候群が，サイトメガロとの関連が否定し切れないとおっしゃっていましたが，今後，側動脈炎やPIE症候群で再燃が見られたときには，ステロイド単

独で治療をされるのか，それともサイトメガロのIgM陽性が続いているということで，今後はガンシクロビルの使用も考えていらっしゃるのかをお聞かせください。

演者　IgMのみでサイトメガロがかかわっているかどうかの判断は難しいと思います。アンチゲネミア等，諸々の検査を行った上で検討したいと思います。

越智　もう1点は，病理組織所見の箇所でサイトメガロのDNAで判定をされたと言われましたが，普通は封入体で見ると思います。封入体と比べ，そのDNAの特異度や感度がどの程度かについて教えてください。

演者　特異度，感度に関しては，調べておりません。

越智　それが陽性であれば，サイトメガロ腸炎を強く疑うということでよろしいのでしょうか。

演者　と，解釈しております。

越智　サイトメガロの腸炎だと，アンチゲネミアが陽性になることは少ないと思います。今後，側頭動脈炎が再燃したときには，やはりもう1回内視鏡を行い，DNAを判定するということになりますか。

武井（共同演者）　先生がおっしゃるように，サイトメガロウイルスの感染かどうか，それが原因かどうかということは，バイラルロードをリアルタイムPCRで測り，そのバイラルロードの数によって判定する方法があります。この症例のときには，まだリアルタイムPCRが私たちのところで可能ではなかったのと，保険適用でないことから検討しておりません。ただ，今後はバイラルロードを測り，その可能性を判断して治療の選択を行うことになるかと思います。

ヘルペスウイルス感染症の制御—Epstein-Barr ウイルス感染と免疫機構を支える遺伝子 (SAP/SH2D1A)

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総　説　特集：ウイルス感染症の制御をめざして

ヘルペスウイルス感染症の制御—Epstein-Barr ウイルス感染と免疫機構を支える遺伝子 (SAP/SH2D1A)

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Herpes Virus Infection, Epstein-Barr Virus, Signaling Lymphocytic-Activation Molecule associated protein (SAP/SH2D1A)

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Herpes virus group viruses include cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus 1 or 2 (HSV), varicella zoster virus (VZV), and human herpes virus-6, 7, and 8. These herpes viruses are transmitted by human contact, cause primary infection, and may exist for several years in a latent state in healthy individuals. These viruses may be reactivated by the dysregulation of the host immune system or possibly by virus mutation. In the present paper, we describe the treatment and diagnosis of HSV, VZV, CMV, and EBV infection and discuss possible future treatments. Our proposed therapy is based on SAP (signaling lymphocytic-activation molecule associated protein) or SH2D1A (Src homology 2 domain-containing protein). SAP (or SH2D1A), an adaptor-like molecule expressed in immune cells, is composed almost exclusively of a Src homology 2 (SH2) domain. In humans, SAP is mutated and either absent or nonfunctional in X-linked lymphoproliferative (XLP) syndrome (Duncan disease), a disease characterized by an inappropriate response to EBV infection. SAP is essential for late B cell help and the development of long-term humoral immunity. The regulation of this molecule (SAP) could become the basis of a new therapeutic approach to the treatment of infections caused by the herpes virus group.

Key words: signaling lymphocytic-activation molecule associated protein (SAP)
ヘルペスウイルス感染症，Epstein-Barr ウイルス

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I. 臨床的意義

近年，小児期での生活衛生環境の改善などから成人でのヘルペスウイルス感染の重症化，HIV 感染症，臓器移植や抗癌剤治療，膠原病リウマチ疾患などへの免疫抑制薬の使用によるヘルペスウイルス関連疾患の発症が日常の臨床の場でも直面することが多くなっている．例えば移植後ウイルス感染症の多くは移植後 180 日以内に生じる．時期は，3 つに区分することができ，移植後 0–30 日の早期は単純ヘルペスウイルス (HSV: Herpes simplex virus) の再活性化が生じる．30–180 日の中間期は，ドナーの臓器，血液製剤によるウイルス再活性化がある．インフルエンザウイルス，RS ウイルス (Respiratory syncytial virus)，アデノウイルス，パラインフルエンザウイルスなどの市中ウイルス感染症や EB ウイルス (EBV: Epstein Barr virus)，サイトメガロウイルス (CMV: Cytomegalovirus) 水痘・帯状疱疹ウイルス (VZV: Varicella zoster virus) はこの時期に発生が一度ピークとなる．また，180 日以降の後期にも CMV 感染症は再び発生のピークがある．この時期に EBV 関連の移植後リ

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ンパ球増殖性疾患 (PTLD: post-transplant lymphoproliferative disorder) も見られる[1]. この稿では臨床の場で遭遇する機会の多いヘルペスウイルス群の HSV, CMV, EBV, VZV について解説する.

II. ウイルスの特徴 ヘルペスウイルス群

ヘルペスウイルスは二本鎖 DNA ウイルスで, 20面体構造を形成し, 細胞膜由来のエンベロープを有する. 潜在感染し, 時に再活性化しウイルスを再産生することがある. HSV, VZV は神経根に潜伏感染し, リンパ球に EBV と CMV は潜在感染する.

A. HSV

HSV には2つの型があり, HSV-1 は主に口腔と結膜, HSV-2 は性器ヘルペスを起こす. 高熱, 嚥下障害, 有痛性の歯肉口内炎が特徴的で, 歯肉は潰瘍を形成することもある. 頬口蓋粘膜, 舌に水疱性病変をきたし, 急速に3～5日間でビランを形成し10日位で瘢痕化する. 所属リンパ節の腫大, 角膜, 口唇周囲皮膚に及ぶことがある. 内蔵病変としては肝炎が最も多く髄膜炎, 脳炎, 肺炎も起こす.

B. CMV

CMV は DNA ウイルスで, 日本人の 90%以上が小児期に感染する. 感染後体内に潜在化し, 免疫抑制状態で再活性化されることがある. CMV は, HIV 感染症, 固形臓器移植後の最も多いウイルス感染症で予後に影響を与える. 移植後では1～3カ月の間に発症するが, 免疫抑制薬の維持量決定後の6カ月以降にも発症する可能性がある. 症状は, 発熱, 間質性肺炎, 肝炎, 脈絡膜炎, 単核球症, 大腸炎があり, 時には消化管に潰瘍を起こし消化管出血を来すこともある. 間質性肺炎は, 生命の予後を左右する. CMV 感染は, 初感染と再活性化がある.

C. EBV

EBV は伝染性単核球症の原因として知られ, 主にBリンパ球に感染し, Burkittリンパ腫や上咽頭癌を引き起こす. その他にも EBV 関与の腫瘍は多く報告されており, Hodgkin 病, B-lymphoproliferative disease, Oral hairy leukoplakia, EBV 関連胃癌などがある. 日本人成人では80%以上既感染となる. EBV は固形臓器移植後の重要な病原体であり, 単核球症, リンパ腫, PTLD を引き起こす. PTLD は, 術後1年目に起こるが, 移植後数年で起こる遅発例もある. 関節リウマチや多発性硬化症, 小児の EBV の重篤な感染を特徴とする免疫不全症候群 (X 連鎖リンパ球増殖症候群：XLP または, Duncan 病) などや慢性活動性 EBV 感染症も知られており, 重篤な血球貪食症候群を起こすこともある. 通常の急性感染症状は発熱, 浸出性扁桃炎, リンパ節腫大, 肝脾腫, 白血球減少, 異型リンパ球などがある.

D. VZV

VZV は初感染では空気感染により水痘として発症し, 局所リンパ節で増殖後, 肝脾臓などで増殖する. その後全身の皮膚の毛細血管内皮細胞や皮膚上皮細胞で増殖する. 夏での水痘の流行は少ない. 水疱を形成し, 膿疱, 痂皮化して治癒する. 水痘罹患後知覚神経から三叉神経節や脊髄後根神経節神経細胞で潜伏感染する. 再活性化されたウイルスは皮膚の神経分布領域に水疱を形成し帯状疱疹となる. 帯状疱疹後神経痛 (post herpetic neuralgia, PHN) が時に生じる. 外耳道炎, 顔面神経麻痺, 耳鳴, 難聴, めまいを症状とする耳の帯状疱疹に Ramsay-Hunt 症候群がある. 帯状疱疹は5日ほどで痂皮形成する. 痂皮になるまで人に伝染能力がある. 再発率は4%以下である.

III. 診断法・治療法 (Table 1)

A. HSV

a. 診断

病変部の細胞診断で非特異的な風船状の細胞が認められる. ウイルス分離のため48時間の組織培養をする. 初感染ではペア血清による抗体価の上昇を見る. IgM 抗体が上昇していれば再活性化, 初感染の証拠となる.

b. 治療

1. アシコルビン (ゾビラックス®)

アシコルビルはヘルペス感染症の第一選択である. 眼部病変には眼軟こうが用いられるが, 口唇口腔, 性器病変や脳炎髄膜炎など内蔵病変には全身投与が行なわれる. 重症時では薬用量 5–10 mg/kg/8 hr で, 静注で7日間とする. 皮膚粘膜ヘルペスは, 1000 mg を1日5回で5日間経口投与する.

2. ビダラビン (アラセナーA®)

ヌクレオシド誘導体の抗ウイルス剤. ビダラビンは HSV 脳炎, 皮膚感染症, 口の感染症, 内蔵感染症に対して使用されている. 局所の皮疹のみでは軟膏の使用で効果があり, 脳炎では 10–15 mg/kg で1日1回7～10日間点滴静注する.

B. CMV

a. 診断

CMV の診断は, HSV と同じく血清抗体検査, 培養によるウイルス同定(シェルバイヤル法), 組織内のウイルス DNA の存在を調べるための単クローン性抗体による感染細胞の検出 (アンチゲネミヤ法), polymerase chain reaction (PCR) 法, in situ ハイブリダイゼーション法や組織, 塗抹組織でウイルスの封入体を示すことが必要である. ウイルス培養では無症候性に CMV を排出するため非特異的に陽性となる場合があり病理組織検査は重要である. 再活性化は抗 CMV-IgM 抗体検査が有効である.

Table 1 Therapy of Herpes virus infection

作用	一般名	商品名	一般的な使用法	副作用	禁忌
抗ヘルペスウイルス薬 ウイルスのDNAポリメラーゼを選択的に阻害する.	ビダラビン	アラセナーA	単純ヘルペス脳炎でアシクロビル無効時1日10～15 mg/kgを10日，免疫抑制患者の帯状疱疹1日5～10 mg/kgを5日間5%糖，生食500 mlを40度以上に加温後，その10 mlをとり300 mgを加え15秒振盪し懸濁液をもとの輸液に戻し40度以上で5分間時々振盪溶解させ冷やしてから使用する.	精神神経障害　骨髄機能抑制 ショック	過敏症既往
	ガンシロクロビル	テノシン	AIDSのサイトメガロウイルス網膜炎に3000 mgを1日3回でまたは，3時間毎1日6回AIDSでサイトメガロウイルス網膜炎の予防3000 mg 1日3回で経口投与. 重篤な場合5 mg/kgを12時間毎1日2回14日間点滴維持療法で5 mg/kgを1日1回7日間.	チエナムと併用しない(けいれん) 消化管出血　骨髄造血能低下 深在静脈血栓症　腎不全　敗血症 膵炎　昏睡錯乱　けいれん	妊婦　好中球数500/mm³未満または血小板数25000/mm³未満の患者　ガンシクロビルまたは類似化合物(アシクロビル等)に対する過敏症
	アシクロビル	ゾビラックス	発病初期5日以内　内服　単純ヘルペス，100 mgを1日5回　5日まで　骨髄移植時単純ヘルペス予防　1000 mgを1日5回で移植7日前から移植後35日まで. 帯状疱疹は4000 mgを1日5回，7日まで. 重症時1回5 mg/kgを8時間毎で1日3回　7日間点滴静注する.	急性腎不全　ショック　血小板減少性紫斑病　DIC呼吸抑制 間質性肺炎　けいれん　幻覚 妄想　てんかん発作	本剤薬剤過敏症　妊婦
	塩酸バラシクロビル	バルトレックス	3000 mgを1日3回　帯状疱疹皮疹出現5日以内　7日投与	アシコルビルに準ず	本剤薬剤過敏症　妊婦
	フォスカーネット	ホスカビル	AIDSでのCMV網膜炎でガンシクロビルが無効または使用できない場合，フォスカーネット60 mg/kgを8時間おきに14–21日間静注し，90 mg–120 mg/kg 1日1回点滴静注で維持する.	腎障害　血清K, Mg, Ca, Na低下 口周囲しびれ　知覚異常　けいれん　横紋筋融解　心不全　血栓性静脈炎　イレウス　敗血症	ペンタミジン投与患者

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b. 治療

アシクロビル，ガンシクロビルやフォスカーネットのような抗ウイルス剤，高力化免疫グロブリン製剤によるCMVの予防，治療は，臓器移植患者の生存率を改善した．

1. アシクロビル (ゾビラックス®)

CMV感染症の予防にアシクロビル大量療法が効果があるとの報告がある．例えば，腎臓移植後に，3200 mg/日のアシクロビルあるいは偽薬を経口で投与し，アシクロビルの有効性が高かったとの報告や，骨髄移植患者にCMV予防としてアシクロビル500 mg/m^{2}を8時間毎に点滴静注したほうが移植後100日以内の生存期間がよかったなどである[2,3]．

2. ガンシクロビル (デノシン®)

グアニンの誘導体で，アシクロビルの構造と類似している．5 mg/kgを12時間毎に点滴し，14～21日間続ける．維持療法は5 mg/kg 1日1回を週7回必要である．CMV網膜炎については眼注による直接投与も考えられている．アルカリ性が強く注意を要する．抗CMV高力化免疫グロブリン2.5～5 g/日，3日間の点滴静注を併用することもある．ガンシクロビルの経口投与 (1000 mgを1日3回，28日間) が，維持治療として検討されている．

3. フォスカーネット (ホスカビル®)

ガンシクロビルが無効または使用できない場合，フォスカーネット60 mg/kgを8時間おきに14～21日間点滴静注し，90 mg–120 mg/kg 1日1回点滴静注で維持する．

C. EBV

a. 診断

EBVの抗体検査は種類があり，それぞれに意味がある．抗ウイルスカプシド抗体IgG, IgM (VCA: Viral Capside Antigen) が有り，IgM抗体が最初出現し，IgG抗体が上昇し存続する．一時的に抗ウイルス早期抗原抗体 (EA: Early Antigen) が認められ，抗EBV関連核蛋白抗原 (EBNA: EBV Associated Nuclear Antigen) 抗体が検出される．抗EBV-VCA IgM抗体が陽性で，抗EBNA抗体が陰性の場合は，初感染を示す．EBVの再活性化では，抗EBV-VCA IgG抗体と抗EBV-EA抗体の高値，慢性活動性感染では抗EBNA抗体の低値が認められる．ウイルスの検出には，培養，検体細胞核のEBNAの検出，in situハイブリダイゼーション法 (特にウイルス由来コピー数が多く感度が高いEBER: EBV encoded small nuclear RNA検出法) やPCR法によるウイルスゲノムの検索が行なわれる．末梢血の異型リンパ球やPaul-Bunnel反応はEBV感染の診断の参考になる．

b. 治療

EBV関連PTLD患者には，重症度により免疫抑制剤の減量，抗ウイルス剤，インターフェロン，化学療法も一般に用いられるが，一定の結論は出ていない．最近の報告ではEBV特異的細胞障害性T細胞を誘導してPTDの治療が試みられている[4]．原則的にはEBV感染症は無治療だが，重篤な嚥下困難や溶血性貧血，血球貪食症候群などがある場合には，副腎皮質ステロイド薬やエトポシド (ラステット®)[5] などの化学療法などが考えられるが，今後の検討が待たれる．

D. VZV

a. 診断

診断は特徴的な臨床経過と皮疹から比較的容易になされる．診断に確信がもてない場合はペア血清による抗体価の動きか再活性化の場合はIgMクラスの抗体の上昇をみて判断する．水疱内にウイルスが存在するためウイルスの分離も必要なら施行する．

b. 治療

1. アシコルビン (ゾビラックス®)

アシコルビルはヘルペス感染症の第一選択である．帯状疱疹は経口で4000 mgを1日5回，7日まで．重症時1回 5 mg/kgを8時間毎で1日3回7日間点滴静注する．

2. 塩酸バラシクロビル (バルトレックス®)

3000 mgを1日3回，帯状疱疹皮疹出現5日以内7日経口投与する．

3. 高齢者への水痘ワクチンの接種は帯状疱疹の発症を予防できる．

IV. 将来の展望

生体は体内に進入した外来微生物を記憶し，再度の侵入に対して免疫記憶の機構を駆使して即座に反応して防御を果たしている．この長期免疫機構を支える新しい遺伝子の発見がなされ，近年その機能の解析が報告されつつある．この免疫を長期記憶して液性免疫による感染制御を行っている遺伝子がSAP (signaling lymphocytic activation molecule associated protein: SLAM associated protein) と呼ばれている．この遺伝子の欠損は小児のEBウイルスの致死的な感染症を特徴とする免疫不全症候群 (X連鎖リンパ球増殖症候群: XLPまたはDuncan病) を惹起することで初めて報告された[6]．我々はこの報告に先立ちIgA腎症の疾患特異的遺伝子の解析をFDD (fluoressein differential display法) で網羅的に行った際この遺伝子を発見し，クローニングを行い国内，国際特許の出願 (国際公開番号WO98-24899) をしている．我々は関節リウマチ (RA) で直接的なEBウイルスとの関与を報告してきたが[7]，SAP遺伝子の発現がRA末梢T細胞で低いことを発見し (出願番号PH11-154625)[8]，そのプロモーター領域に変異があることを見出している．

SAPはSH2ドメインのみを有するアダプター蛋白質 (128アミノ酸) で進化の過程で生き残ってきたnatural regulatorともいえるもので，シグナル伝達の制御ポイントとして重要な役割を果たしている．T細胞とNK細胞

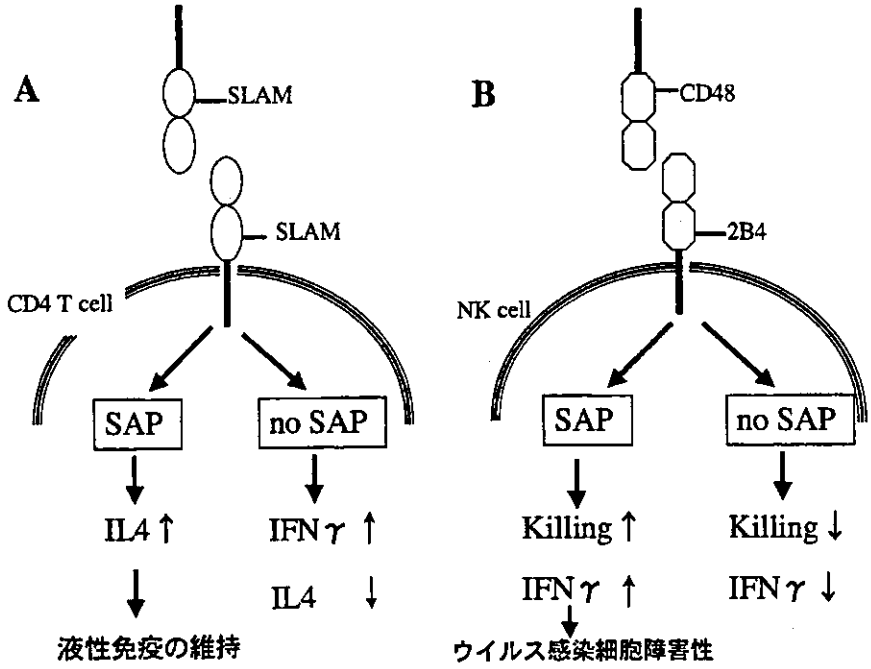

Fig. 1　SAP is essential for development of long-term humoral immunity and killing of viral infected cells.

のみで選択的に発現し，SLAM (signaling lymphocytic activation molecules, CD150)-family receptor (CD2 サブファミリー；SLAM, 2B4 (CD244), LY9 (CD229), CD84, NTB-A) の細胞内リン酸化チロシン (TIYXXV) に結合し，SRC キナーゼのリクルートによる SLAM (CD150)-family receptor のシグナル伝達を制御している[9,10]．SLAM は，麻疹ウイルスのレセプターであり，関節リウマチや多発性硬化症にも関与していることが報告されている．SAP は，細胞内において Dok1 (RAS-GAP, CSK, NCK と相互作用する) とも会合し，NF-κB の活性化に関与していることも報告された[10]．

更に，SAP は長期的な体液性免疫を維持するために必要であることが最近発見された．長期間生存するプラズマ細胞やメモリー B 細胞は長期的な体液性免疫の主要な細胞成分であり，またそれ自体ほとんどのワクチンがもたらす防護作用にとって極めて重要なものである．SAP ノックアウトマウスのモデル系を使って免疫反応における SAP の役割が解析されている．SAP の発現を欠くマウスでは，ウイルス感染の後，激しい急性の IgG 抗体反応は起こるが，ウイルス特異的な長期生存プラズマ細胞およびメモリー B 細胞がほぼ完全に欠失していることが判明している．養子移入実験では，SAP 欠損 B 細胞は正常であり，欠陥は CD4+T 細胞にあることが示されている．SAP は，初期段階における B 細胞に対する補助やクラススイッチには不要であるが，後期段階の B 細胞に対する補助や長期的な体液性免疫の成立には不可欠であることがわかっている．このように SAP は，免疫記憶の発達に中心的な役割を果たしている[11](Fig. 1)．

また，SAP は Th1 への分化を抑制し，Th2 への分化を誘導すると報告された．SAP のノックアウトマウスでは，CD4+T 細胞からの IFN-γ 産生が高まり，いわゆる Th1 細胞への分化が観察された．一方，SAP のトランスジェニックマウスでは，IL-4 産生が高まり，Th2 細胞への分化が認められた[12]．以上から SAP は，ヘルパー T 細胞機能の重要な役割を担っていると考えられる．

また，SAP には dual functional role が報告されており，フォスファターゼ SHP-2 に対して SAP の SH2 ドメインの phosphotyrosine-binding motif を介して SLAM の細胞内ドメインのチロシンリン酸化部位で阻害因子としての役割と SAP の SH2 ドメインの phosphotyrosine-binding motif とは異なる second region を介して FynT の SH3 ドメインと相互作用し，FynT (PTK) の SLAM へのリクルートを促進するアダプターとしての役割 (SLAM-SAP-FynT complex を形成 → FynT のリン酸化機能の構造的なマスキングが解除，リン酸化機能が活性化され下流にシグナル伝達) を果たしている[13~16]．

HSV や CMV などのヘルペスウイルス急性感染症に対する治療は前述したように抗ウイルス薬や免疫グロブリンでの治療が確立されているが，EBV に対する治療法はまだ，確立されたものがなく，様々な EBV 関連疾患治療法は今後の研究が待たれている．

EBV 関連疾患は悪性リンパ腫や胃癌から血球貪食症候群，臓器移植後日和見腫瘍，膠原病 (RA，全身性エリテマトーデス，Sjogren 症候群など) の治療予防に深く関わる可能性があり，XLP (Duncan 病) の疾患原因遺伝子 (SAP) の発見から端を発した液性免疫記憶の機序の解析はこれら疾患の新たな治療の開発につながるものと考えられる．具体的な方法としては SAP 遺伝子を造血幹細

胞や血液細胞に導入する遺伝子治療や SLAM (CD150)-family receptor (SLAM, 2B4, CD48, LY9, CD84) のシグナル伝達を +/− に制御する薬剤 (Th1/2 バランス制御薬), 有効なウイルス薬が無いなかで, ワクチンの効果を維持したり高めたりする薬剤, SAP 機能の促進剤は, ヘルパー T 細胞からの IL-4 の産生を高めナイーブ Th0 細胞の Th2 細胞への分化を誘導し, Th1/Th2 バランスを Th2 へ傾ける薬剤となり, 液性免疫の強化剤, EBV 関連腫瘍の治療薬, 予防薬としての開発が考えられる. 我々がこの感染防御に関わる遺伝子 (SAP) の知的財産権を主張できる立場を世界に先立ち有していることはこの分子を利用した新たな治療法の開発に優位な状況にあると考える.

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Distinct roles of Smad pathways and p38 pathways in cartilage-specific gene expression in synovial fibroblasts

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The role of TGF-β/bone morphogenetic protein signaling in the chondrogenic differentiation of human synovial fibroblasts (SFs) was examined with the adenovirus vector–mediated gene transduction system. Expression of constitutively active activin receptor–like kinase 3 ($ALK3^{CA}$) induced chondrocyte-specific gene expression in SFs cultured in pellets or in SF pellets transplanted into nude mice, in which both the Smad and p38 pathways are essential. To analyze downstream cascades of ALK3 signaling, we utilized adenovirus vectors carrying either Smad1 to stimulate Smad pathways or constitutively active MKK6 ($MKK6^{CA}$) to activate p38 pathways. Smad1 expression had a synergistic effect on $ALK3^{CA}$, while activation of p38 MAP kinase pathways alone by transduction of $MKK6^{CA}$ accelerated terminal chondrocytic differentiation, leading to type X collagen expression and enhanced mineralization. Overexpression of Smad1 prevented $MKK6^{CA}$-induced type X collagen expression and maintained type II collagen expression. In a mouse model of osteoarthritis, activated p38 expression as well as type X collagen staining was detected in osteochondrophytes and marginal synovial cells. These results suggest that SFs can be differentiated into chondrocytes via ALK3 activation and that stimulating Smad pathways and controlling p38 activation at the proper level can be a good therapeutic strategy for maintaining the healthy joint homeostasis and treating degenerative joint disorders.

Introduction

Injury to the articular cartilage occurs under various pathological conditions such as trauma, inflammation, and aging (1), and cartilage injury is followed by osteoarthritic changes of the affected joints. Osteoarthritis is the most common degenerative joint disorder, affecting nearly half of the elderly population. Osteoarthritis is characterized by degradation of articular cartilage and overgrowth of cartilage and bone, known as osteophytes, at the periphery of the articular surface, which results in pain and loss of joint function (1, 2). Microscopically, loss of proteoglycan and fibrillation of the articular surface are observed at the early stage of arthritis. At later stages, clefts are formed, and at the end stage, erosive changes in the articular cartilage appear. The high prevalence of this disease results in high costs for treating patients, and therefore the development of good therapeutics for osteoarthritis is a matter of great urgency. Because of the limited capacity of spontaneous healing, the regeneration of intact articular cartilage is one of the most challenging issues in the orthopedic field (3, 4). Transplantation of autologous chondrocytes or mesenchymal progenitor cells and autogenous osteochondral transplantation (mosaicplasty) have been successfully utilized for the repair of focal osteochondral defects (3, 5–11). However, the application of these technologies is limited to small defects due to the difficulty of obtaining a sufficient amount of cells or tissues.

Synovium is a thin tissue lining the nonarticular surfaces of diarthrodial joints (12). Synovial tissues contain various types of cells, including type A cells, macrophage lineage cells, and type B cells, which are specialized synovial fibroblasts (SFs). It is now widely recognized that synovial tissues are involved primarily in the pathogenesis of arthritic joint disorders such as rheumatoid arthritis by producing the matrix-degenerating enzymes cystein proteases and matrix metalloproteinases (MMPs) and the proinflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) (12). We previously reported that SFs express a high level of receptor activator of NF-κB ligand, the osteoclast differentiation factor belonging to the TNF-α superfamily (13). In contrast to such catabolic actions, there is accumulating evidence that synovial cells have anabolic effects, leading to bone and cartilage production. Hunziker and Rosenburg reported that synovial cells can migrate into partial-thickness articular cartilage defects, where they proliferate and subsequently deposit a scar-like tissue (14). Nishimura et al. (15) demonstrated SFs show chondrogenic differentiation after being cultured in the presence of TGF-β, and de Bari et al. recently demonstrated that multipotent mesenchymal stem cells could be isolated from human synovial tissues, which differentiated into chondrocytes as well as osteoblasts, adipocytes, and myotubes under proper culture conditions (16, 17). Another dramatic clinical manifestation of the chondrogenic potential of synovial tissues is synovial chondromatosis, a tumor-like disorder characterized by the formation of multiple cartilaginous nodules, which is believed to be benign reactive metaplasia of synovial cells (18). These observations

Nonstandard abbreviations used: anterior cruciate ligament (ACL); bone morphogenetic protein (BMP); constitutively active activin receptor-like kinase 3 ($ALK3^{CA}$); constitutively active MKK6 ($MKK6^{CA}$); hemagglutinin (HA); matrix metalloproteinase (MMP); medial meniscus (MM); osteoarthritis (OA); receptor-regulated Smad (R-Smad); synovial fibroblast (SF); TGF-β–activating kinase 1 (TAK1).

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lead us to speculate that synovial tissues contain multipotent cells with chondrogenic potential that might be involved in the repair process of articular cartilage defects and therefore might provide a good source for engineering articular cartilage.

There is accumulating evidence that TGF-β superfamily cytokines play an essential role in bone and cartilage development. Wozney and coworkers (19) reported that bone morphogenetic proteins (BMPs) induce early cartilage formation, and various studies have shown that TGF-β induces chondrocytic differentiation of undifferentiated mesenchymal cells (20–22). In the present study, we analyzed the role of TGF-β/BMP signaling on chondrogenic differentiation of human SFs by using the adenovirus vector-mediated gene transduction system. The introduction of an activated mutant of ALK3 (constitutively active activin receptor-like kinase 3 [$ALK3^{CA}$]), also known as BMP type IA receptor, induced chondrocyte-specific marker expression in the cells. ALK3 signaling involves two different downstream cascades, the Smad pathway and the p38 MAP kinase pathway. We used a combination of adenoviral gene delivery and chemical inhibition to analyze the role of these two signaling cascades in inducing differentiation of SF cells toward a chondrocyte phenotype and found that both pathways are essential for chondrogenic differentiation. Interestingly, activation of p38 pathways alone induced markers of terminal chondrocyte differentiation, type X collagen expression and mineralization, which was suppressed by Smad1 coexpression. These results suggest that both the Smad and p38 pathways are necessary for chondrogenic differentiation of SFs and that the balance between these two pathways determines the stage of differentiation.

Methods

Chemicals and antibodies. Alpha-modified minimum essential medium (α-MEM) was purchased from Gibco BRL, Life Technologies Inc. (Rockville, Maryland, USA), and fetal bovine serum (FBS), from Sigma-Aldrich (St. Louis, Missouri, USA). Anti-p38 MAPK and anti-phospho-p38 MAPK (Thr180/Tyr182) were obtained from Cell Signaling Inc. (Cummings Center, Beverly, Massachusetts, USA). Anti-Flag was purchased from Sigma-Aldrich, and anti-hemagglutinin (anti-HA) was from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Anti-phospho-Smad1/5/8, which recognizes the phosphorylated form of Smad1, Smad 5, and Smad8, and anti-phospho-Smad2 were from Cell Signaling Inc. Anti-type II collagen was purchased from Oncogen (Boston, Massachusetts, USA) and anti-type X collagen was from LSL Co. (Cosmo Bio, Tokyo, Japan). Other chemicals and reagents used in this study were of analytical grade.

Isolation of SFs from human synovial tissues. Synovial cells were obtained as previously described (13, 23, 24). In brief, with enzymatic digestion, human synovial cells were isolated from synovial tissues of the knee joints of ten rheumatoid arthritis patients (37–75 years of age; mean, 60.3 years of age) at the time of total knee arthroplasty operations. Written informed consent for subsequent experiments was obtained from each patient. Cells were suspended in α-MEM containing 10% FBS and were cultured in monolayers. After three to five passages, subcultured cells were composed of morphologically uniform fibroblastic cells (SFs) that were free of macrophages. They were infected with adenovirus vectors and cultured in pellets ("pellet culture"). Primary chondrocytes were obtained from articular cartilage resected during the surgeries. Cartilage was minced finely in phosphate-buffered saline (PBS), and chondrocytes were isolated by sequential digestion at 37°C with 0.25% (weight/volume) trypsin for 30 minutes and with 2 mg/ml of clostridial collagenase in α-MEM containing 10% FBS and antibiotics (penicillin at 100 μg/ml and streptomycin at 100 μg/ml) overnight on an orbital shaker. Cells were isolated by centrifugation and were resuspended in α-MEM with 10% FBS. Cells were cultured in monolayers for 1 day and then subjected to RNA isolation.

Constructs and gene transduction. The recombinant adenovirus vectors carrying various molecules that modulate TGF-β superfamily signaling pathways, that is, HA-tagged constitutively active TGF-β/BMP type I receptors ($ALK3^{CA}$, $ALK5^{CA}$, and $ALK6^{CA}$), constitutively active MKK6 ($MKK6^{CA}$), Flag-tagged Smad1 and Smad6 with CAG [cytomegalovirus IE enhancer + chicken β-actin promoter + rabbit β-globin poly(A) signal] promoter, were generated by the DNA-terminal protein complex method (25–27). SFs were infected with adenovirus vectors following a method previously described (13). In short, subconfluent SFs were incubated with a small amount of medium (α-MEM without serum) that contained the recombinant adenoviruses for 2 hours at 37°C at the indicated multiplicity of infection (MOI) and then with 10 times more medium to which 10% FBS had been added. Infected cells were cultured for additional 3 days for assessment of chondrogenic gene expression or were subjected to pellet culture 24 hours after the infection for histological examination.

Pellet cultures of isolated SFs. After 24 hours of viral infection, adherent cells were trypsinized and cells numbers were ascertained. Aliquots of 5×10^5 cells were spun down at 500 *g* in 15-ml polypropylene conical tubes in 5 ml of α-MEM with ascorbate 2-phosphate (0.1 mM) and 10% FBS. The cells were incubated at 37°C in 5% CO_2. Within 24 hours after incubation, the cells formed a single, free-floating pellet. The medium was changed every 2–3 days, and duplicate pellets were harvested after 3 and 7 days for real-time-PCR and Northern blotting and after 3 and 5 weeks for histological and immunohistochemical analysis. For visualization of the chondrogenic differentiation in vivo pellets were transplanted subcutaneously into *nu/nu* BALB mice (nude mice) after 3 days of pellet culture. Mice were sacrificed 5 weeks after transplantation and the pellets were recovered and subjected to toluidine blue staining as well as immunostaining with anti-type II collagen.

Immunoblotting. All the extraction procedures were performed at 4°C or on ice. Cells were washed with PBS and then lysed by the addition of TNE buffer (1% NP-40, 10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA, 2 mM Na_3VO_4, 10 mM NaF, and 10 μg/ml aprotinin). Lysates were prepared by centrifugation at 10,000 *g* for 20 minutes. An equal amount (15 μg) of proteins was separated by electrophoresis on 10% SDS-polyacrylamide gels. After electrophoresis, proteins were electronically transferred onto a nitrocellulose membrane. Immunoblotting with specific antibodies was performed with ECL Western blotting reagents (Amersham Co., Arlington Heights, Illinois, USA) according to the conditions recommended by the supplier.

Histology and immunostaining. Pellet cultures were fixed with 3.7% formaldehyde, embedded in paraffin, and cut into sections 4 μm in thickness. Representative sections were subjected to Alcian blue staining, Alizarin red staining, and immunohistochemistry. Alcian blue staining was performed according to the protocol described previously (28). Briefly, after deparaffinization, sections were stained with 0.5% Alcian blue 8GX (Wako, Osaka, Japan) in 0.1 N HCl for 1 hour. Mineralization was assessed by Alizarin red staining. In brief, sections were immersed in Alizarin red solution (40 mM, at pH 4.0) for 8 minutes at room temperature, and nonspecific staining was removed by several washes in distilled water. For immunostaining with anti-type

II collagen or anti-type X collagen, we utilized a CSA Kit (DAKO, Carpinteria, California, USA) following the manufacturer's protocol.

Total RNA extraction and real-time PCR. Total RNA was isolated from SFs with ISOGEN (Wako) following the supplier's protocol. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA with the Superscript II reverse transcriptase kit (Invitrogen, Carlsbad, California, USA). For real-time PCR, the ABI Prism Sequence Detection System 7000 was used. Primers were designed based on sequences obtained from GenBank and amplicons of 50–250 base pairs with a melting temperature of between 55°C and 60°C were selected. Aliquots of first-strand cDNA (1 μg) were amplified with the QuantiTect SYBER Green PCR Kit (Qiagen, Valencia, California, USA) under the following conditions: initial denaturation for 10 minutes at 94°C followed by 40 cycles consisting of 15 seconds at 94°C and 1 minute at 60°C. Data analysis consisted of fold induction, and the expression ratio was calculated from the differences in threshold cycles at which an increase in reporter fluorescence above a baseline signal could first be detected among three samples and was averaged for duplicate experiments. The primers we utilized in real-time PCR to detect sox9, type II collagen, type X collagen, osteocalcin, osteopontin, and GAPDH were as follows: sox9, 5′-AGAAGGACCACCCGGATTAC-3′ and 5′-AAGTCGATAGGGGGCTGTCT-3′; type II collagen, 5′-GGTGGCTTCCATTTCAGCTA-3′ and 5′-TACCGGTATGTTTCGTGCAG-3′; type X collagen, 5′-AGGAATGCCTGTGTCTGCTT-3′ and 5′-ACAGGCCTACCCAAACATGA-3′; osteocalcin, 5′-GTGCAGAGTCCAGCAAAGGT-3′ and 5′-CGATAGGCCTCCTGAAAGC-3′; osteopontin, 5′-ACAGCCAGGACTCCATTGAC-3′ and 5′-ACACTATCACCTCGGCCATC-3′; and GAPDH, 5′-GAAGGTGAAGGTCGGAGTCA-3′ and 5′-GAAGATGGTGATGGGATTTC-3′.

Northern blotting. Equal amounts (15 μg) of RNA were denatured in formaldehyde, separated by 1% agarose gel electrophoresis and transferred to a nitrocellulose membrane (Hybond N+) (Amersham Pharmacia, Piscataway, New Jersey, USA), followed by ultraviolet crosslinking. ULTRAHyb hybridization solution (Ambion, Austin, Texas, USA) was used according to the manufacturer's protocol. The blots were hybridized with a cDNA probe labeled with [α-^{32}P]dCTP using Ready-To-Go DNA Labeling Beads (Amersham Pharmacia). Rabbit type II collagen and aggrecan probes were generously provided by Yoshiyasu Iwamoto (Thomas Jefferson University, Philadelphia, Pennsylvania, USA). Membranes were washed in 2× SSC for 15 minutes at 42°C and then in 0.1× SSC for 30 minutes at 65°C. For visualization, X-ray film was exposed to membranes overnight at -80°C.

Osteoarthritis model mice. Osteoarthritic changes were developed in the knee joint by transection of the anterior cruciate ligament (ACL) and medial meniscus (MM) in C57BL/6 mice (mean age, 8 weeks) (29, 30). Briefly, after mice were anesthetized with ketamine and xylazine, a medial parapatellar skin incision was made. The subcutaneous tissues were incised and retracted, along with the articular capsule. The medial compartment of the knee joint was visualized and the ACL and MM were transected with a scalpel, and thereafter the capsule, medial retinaculum, and skin were sutured. Mice were housed in regular individual cages and allowed to exercise. Eight weeks after the surgery, the mice were sacrificed and paraffin-embedded sections of the affected joints were immunostained with anti-type X collagen and anti-phospho-p38 (Cell Signaling Technology Inc).

Results

Adenovirus-mediated gene transduction modulates the Smad and p38 pathways in SFs. We previously reported that adenovirus vectors can efficiently transduce foreign genes into synovial cells both in vitro and in vivo and that adenovirus infection itself does not affect the phenotypes of the cells (13). We constructed adenovirus vectors to analyze the role of ALK signaling as well as the Smad pathways and p38 pathways, which lie downstream of ALK signaling. SFs were infected with adenovirus vectors carrying various signaling molecules that modulate TGF-β superfamily signaling pathways, that is, HA-tagged constitutively active ALK3, ALK5, and ALK6 constitutively active MKK6, and Flag-tagged Smad1 and Smad6, as well as a control virus carrying the β-galactosidase gene (LacZ virus), and gene expression was determined by immunoblotting with specific antibodies. As shown in Figure 1, clear induction of the genes encoding ALK3^{CA}, ALK5^{CA} and ALK6^{CA} was observed by immunoblotting with anti-HA (Figure 1A), and Smad1 and 6, by anti-Flag (Figure 1B). ALK3^{CA} or ALK6^{CA} overexpression induced phosphorylation of Smad1, Smad5, and Smad8 in SFs, and ALK5^{CA}-transduced cells showed Smad2 phosphorylation (Figure 1A). MKK6^{CA} virus infection specifically activated p38 pathways in SFs, and the pathways were also activated in ALK3^{CA}-transduced cells as determined by Western blotting with anti-phospho-p38 (Figure 1C). The increased p38 phosphorylation induced by either ALK3^{CA} or MKK6^{CA} overexpression was suppressed by the p38-selective inhibitor SB203580.

Induction of chondrocyte-specific gene expression by ALK3^{CA} transduction in pellet cultures of SFs. To determine the effects of these transduced gene products on chondrocyte-specific gene expression in SFs, we subjected infected cells to pellet culture. After 7 days of culture, clear induction of type II collagen and aggrecan genes was observed in ALK3^{CA}-transduced cultures by both Northern blot analysis (Figure 2A) and real-time PCR (Figure 2, B and C). Expression of these genes was also observed in ALK6^{CA}-transduced cultures, albeit less efficiently, as shown in Figure 2, B and C, by real-time PCR. Contrary to the strong chondrogenic effects of ALK3^{CA} virus, expression

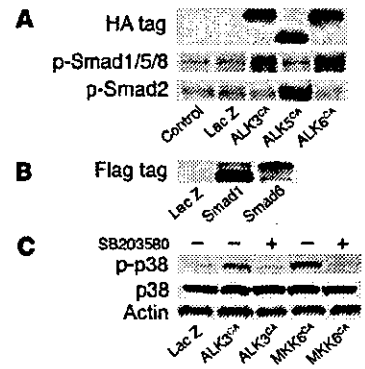

Figure 1
Modulation of intracellular signaling pathways by adenovirus vector–mediated gene transduction into SFs. (**A**) SFs at passage 3 were transduced with HA-tagged constitutively active ALK3, ALK5, and ALK6, and the expressed products were detected by immunoblotting after 2 days of viral infection. Expression of these genes was detected by immunoblotting with anti-HA and phospho-Smad1, -Smad 5, and Smad8 (p-Smad1/5/8) was observed in cells expressing ALK3^{CA} or ALK6^{CA}, and p-Smad2, in cells expressing ALK5^{CA}. (**B**) Expression of Smad1 and 6 in SFs was determined by anti-Flag. (**C**) Adenovirus vector–mediated ALK^{CA} or MKK6^{CA} expression specifically activated p38 pathways in SFs, as determined by Western blotting with anti–phospho-p38 (p-p38). The increased p38 phosphorylation induced by ALK3^{CA} or MKK6^{CA} overexpression was suppressed by the p38-selective inhibitor SB203580.

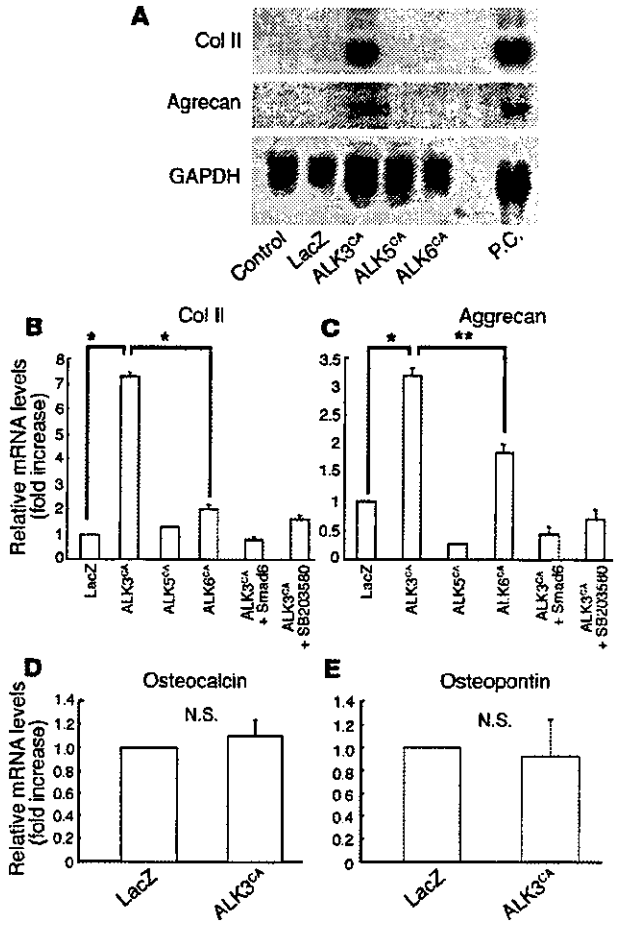

Figure 2
Effects of ALK3^{CA}, ALK5^{CA}, and ALK6^{CA} expression on chondrocyte-specific gene expression in SFs. (**A–E**) Gene expression in SFs, as determined by Northern blot analysis (**A**) and real-time PCR analysis (**B–E**). Subconfluent monolayer SF cultures were infected with adenovirus vectors and they were then subjected to pellet culture 24 hours after viral infection; mRNA extracted from the pellets after 7 days of culture was then analyzed. Expression of type II collagen (Col II) and aggrecan was clearly induced in ALK3^{CA}-expressing cultures, as shown by Northern blot analysis (**A**) and real-time PCR analysis (**B** and **C**); this was suppressed by Smad6 coexpression and SB203580 (**B** and **C**). Expression of type II collagen and aggrecan was also observed in ALK6^{CA}-expressing cultures, albeit less efficiently, as shown in **B** and **C** by real-time PCR. Neither the osteocalcin nor the osteopontin gene was induced by ALK3^{CA} virus infection (**D** and **E**). P.C., positive control, which represents the Northern blotting using mRNA of primary chondrocytes. N.S., not significant; $^{*}P < 0.001$; $^{**}P < 0.005$ (significantly different).

of osteocalcin or osteopontin was hardly detectable in the cells (Figure 2, C and D), indicating that hypertrophic and osteogenic differentiation were somehow blocked in these cultures. In contrast, neither type II collagen nor aggrecan gene expression was observed in ALK5^{CA} virus-infected cells (Figure 2, A–C). Type II collagen and aggrecan expression induced by ALK3^{CA} transduction was completely suppressed by coexpression with Smad6 or by SB203580 (Figure 2, B and C).

ALK3 gene transduction increases Alcian blue–positive matrix and type II collagen deposition in pellet cultures of SFs. For histological analysis, cells were subjected to pellet culture 24 hours after the viral infection. After 3 weeks of pellet culture, cells were fixed and examined by Alcian blue staining (Figure 3, A, D, G, and I) and Alizarin red staining and type II collagen immunostaining (Figure 3, B, E, G, and J) and type X collagen immunostaining (Figure 3, C and F). ALK3^{CA} virus-infected cultures showed cartilageous matrix production that was strongly positive for Alcian blue staining (Figure 3D), while no positive staining was observed in LacZ virus-infected cultures (Figure 3A) or ALK5^{CA} virus-infected cultures (Figure 3G), and only weak staining was observed in ALK6^{CA} virus-infected cultures (Figure 3H). No Alizarin red staining was observed in ALK3^{CA}-infected cultures (not shown), indicating that mineralization associated with osteogenic differentiation was not induced. ALK3^{CA} virus-infected SFs showed an oval shape, morphologically reminiscent of chondrocytes (Figure 3D). Immunostaining with anti-type II collagen showed positive staining in ALK3^{CA} virus-infected pellet cultures (Figure 3E) and weak staining in ALK6^{CA} virus-infected cultures (Figure 3H), while we failed to detect type X collagen in ALK3^{CA} virus-infected cultures (Figure 3F), which suggests an absence of terminal differentiation to hypertrophic chondrocytes. No positive type II collagen immunostaining was detected in LacZ virus-infected cultures (Figure 3B) or ALK5^{CA} virus-infected cultures (Figure 3H).

ALK3^{CA}-transduced SFs after pellet culture form cartilage matrix in vivo. To study chondrogenic differentiation of SFs in vivo, we subcutaneously transplanted the pellets into nude mice. Mice were sacrificed 3 weeks after the transplantation and the pellets were recovered and subjected to histological analysis. The transplanted SF pellets expressing ALK3^{CA} were positively stained for toluidine blue (Figure 4C), which detects proteoglycan components, as does Alcian blue staining. Type II collagen immunostaining was also positive (Figure 4D), indicating the cartilaginous differentiation of the cultures in vivo, while Alizarin red staining was almost undetectable (data not shown). ALK6^{CA} expression also induced chondrogenesis, albeit much less prominently (not shown), while neither LacZ (Figure 4, A and B) or ALK5^{CA} (not shown) expression could induce chondrogenic phenotypes in the cultures. The histological observation was further confirmed by real-time PCR; expression of type II collagen and aggrecan was significantly higher in ALK3^{CA}-transduced pellets (Figure 4, E and F). These results suggest that ALK3^{CA}

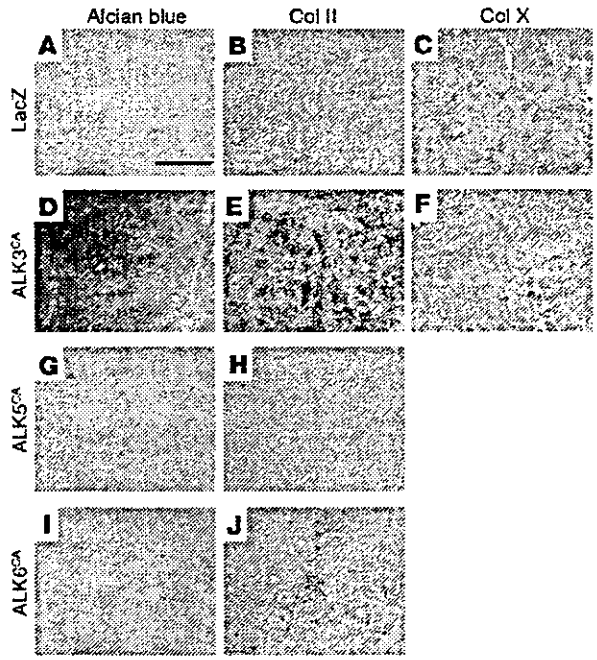

Figure 3
ALK3^{CA} gene transduction increases Alcian blue–positive matrix and type II collagen deposition in pellet cultures of SFs. (**A–J**) Adenovirus-infected SF pellets were fixed with 3.7% formaldehyde after 3 weeks of culture and then were subjected to Alcian blue staining (**A**, **D**, **G**, and **I**) or immunostaining with anti–type II collagen (**B**, **E**, **H**, and **J**) or anti–type X collagen (Col X) (**C** and **F**). Distinct Alcian blue (**D**) and type II collagen (**E**) staining was observed in ALK3^{CA}-expressing cultures. ALK6^{CA}-expressing cultures showed weaker staining (**I** and **J**), and no positive staining was observed in ALK5^{CA} virus–infected (**G** and **H**) or LacZ virus–infected (**A** and **B**) cultures. No type X collagen immunostaining was observed in cultures expressing LacZ or ALK3^{CA} (**C** and **F**). Scale bar: 100 μm.

overexpression was able to target cartilage formation without subsequent bone formation in vivo.

Segregation of ALK signaling pathways. ALK signaling is known to be mediated by both the Smad pathways and MAP kinase pathways, especially the p38 pathways (31–33). We therefore attempted to distinguish the roles of the Smad pathways and p38 pathways from each other using a specific p38 inhibitor or adenovirus vectors. Smad6 coexpression or treatment of the cultures with the p38 inhibitor SB203580 completely abrogated the chondrogenic gene expression induced by ALK3^{CA} (Figure 2, B and C). These results indicate that both the Smad pathways and the p38 MAP kinase pathways are required for the differentiation. Although Smad1 expression alone (MOI = 20) or a small amount of ALK^{CA} virus (MOI = 2) failed to induce type II collagen expression in SFs, both had synergistic effects, and robust upregulation of type II collagen gene was observed by coinfection of Smad1 virus (MOI = 20) and ALK3^{CA} virus (MOI = 2) (Figure 5A). Interestingly, activation of p38 pathways alone by MKK6^{CA} expression in SFs induced rapid induction of Sox9 and type II collagen, which declined rapidly, however, and type X collagen expression was subsequently increased (Figure 5B). Coexpression of Smad1 together with MKK6^{CA} not only reduced type X collagen expression but also maintained type II collagen expression in the cells (Figure 5B). Pellet cultures infected

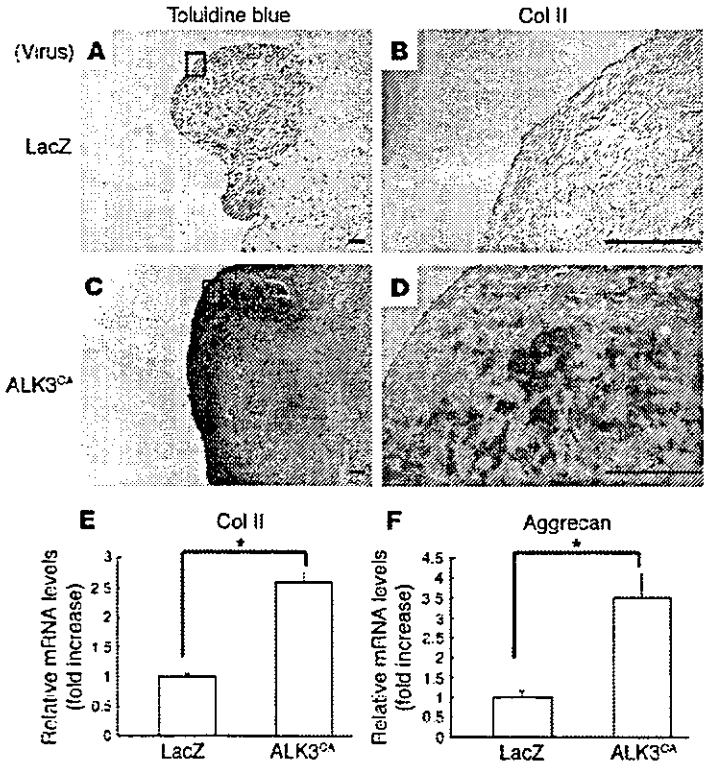

Figure 4
ALK3^{CA}-transduced SFs form cartilage matrix in vivo. (**A–D**) Three weeks after transplantation into nude mice, pellets were recovered and stained with toluidine blue (**A** and **C**) and immunostained with anti–type II collagen (**B** and **D**). Type II collagen immunohistochemistry was shown in the enlarged features of the rectangular area in the toluidine blue staining. Distinct positive staining was observed in ALK3^{CA}-expressing cultures (**B** and **D**) in contrast to LacZ virus–infected cultures (**A** and **C**). Scale bars: 100 μm. (**E** and **F**) Real-time PCR analysis of type II collagen and aggrecan. Their expression was significantly higher in ALK3^{CA}-expressing pellets than in LacZ-expressing pellets. *$P < 0.001$ (significantly different).

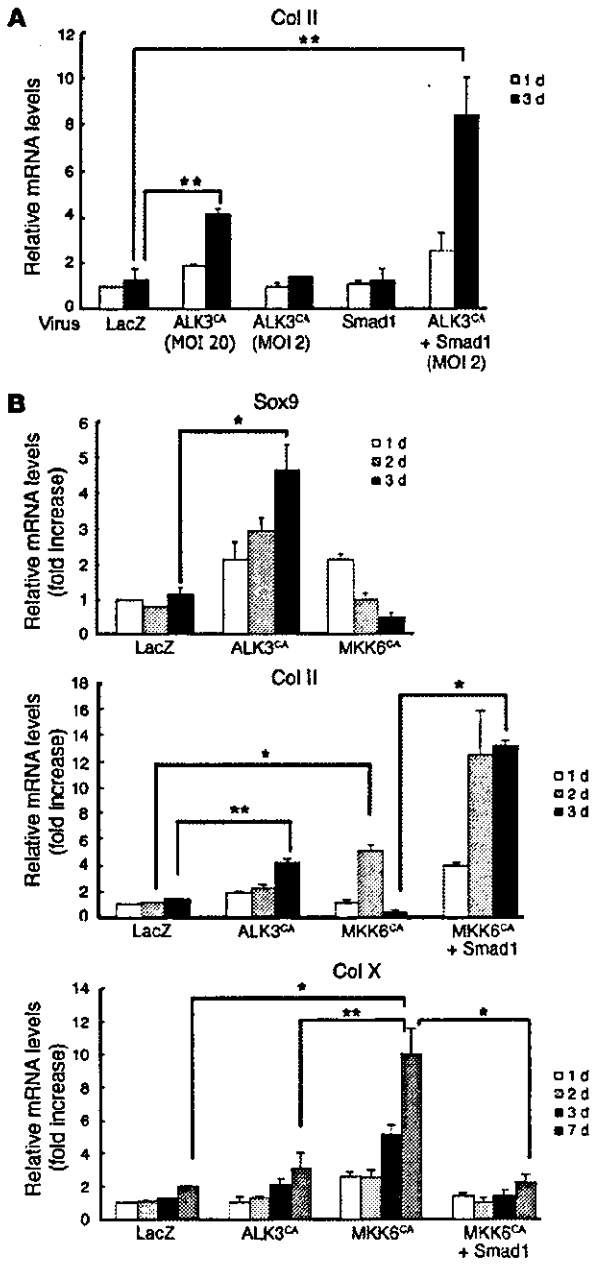

Figure 5
Segregation of downstream signaling pathways of ALK3. (**A**) Synergistic effect of Smad1 expression on the chondrogenic effects of the ALK3^{CA} virus. Expression of Smad1 (MOI = 20) together with ALK3^{CA} virus (MOI = 2) strongly induced expression of type II collagen in SFs. White bars indicate type II collagen expression on day 1 of cultures, and black bars indicate that on day 3. $^{**}P < 0.005$ (significantly different). (**B**) MKK6-p38 pathways promote terminal chondrocytic differentiation of SFs. Mandatory activation of p38 pathways by expression of MKK6^{CA} using adenovirus vectors rapidly activated expression of the Sox9 and type II collagen genes, which rapidly declined, while expression of a terminal chondrocytic differentiation marker, type X collagen, was gradually increased. Adenovirus vector–mediated overexpression of Smad1 together with MKK6^{CA} suppressed type X collagen expression and maintained type II collagen expression in SFs. $^{*}P < 0.001$; $^{**}P < 0.005$ (significantly different).

with MKK6^{CA} virus were positively stained by type X collagen immunostaining as well as Alizarin red staining, which was suppressed by Smad1 virus coinfection (Figure 6).

Type X collagen expression and p38 activation in synovial cells in osteoarthritic joints. To examine the role of p38 activation in the development of degenerative changes in the articular cartilage, we next analyzed synovial tissues in the mouse model of osteoarthritis. After ACL and MM resection, the animals developed degenerative joint changes mimicking osteoarthritis. Osteochondrophytes were formed at the posterior edge of the femoral condyle and they were positively stained by anti-type X collagen as well as toluidine blue (rectangular areas in Figure 7, A and C) 4 weeks after the operation (corresponding to the stage of moderate osteoarthritis). Clusters of migrating synovial cells were observed adjacent to the osteochondrophytes (Figure 7B, arrowheads), where future osteochondrophytes will develop, and they were weakly stained by toluidine blue and anti-type X collagen at the marginal area between synovium and osteophytes (rectangular areas in Figure 7, B and D). This region was also positively stained by anti-phospho-p38 (Figure 7F). No positive staining was observed in the normal synovium, however (data not shown).

Discussion

The signaling events leading to chondrogenesis still remain elusive, although there is accumulating evidence that TGF-β superfamily cytokines may play an important role (19–22). The receptors of TGF-β family members are composed of two different types of serine/threonine kinase receptors, known as type I and type II (31, 34, 35). Type II receptors are constitutively active kinases and phosphorylate type I receptors, also called ALKs. Type I receptors in turn mediate specific intracellular signaling pathways and therefore determine the specificity of the downstream signaling. So far, seven type I receptors have been identified, ALKs 1–7. ALK3 (BMPR-IA) and ALK6 (BMPR-IB) are structurally similar to each other and function as BMP receptors, while ALK5 and ALK4 work as type I TGF-β receptors. Using the adenovirus vector system, Fujii et al. reported that ALK1^{CA}, ALK2^{CA}, ALK3^{CA}, and ALK6^{CA} induced osteoblastic differentiation of C2C12 myoblasts and that ALK3^{CA} or ALK6^{CA} introduction induced chondrocytic differentiation of ATDC teratocarcinoma cells (27).

In the present study, we focused on the regulation of chondrogenic differentiation of primary SFs obtained from rheumatoid arthritis patients. SFs have chondrogenic potential (15, 16) and can migrate into articular cartilage defects, where they deposit a scar-like tissue as Hunziker et al. pointed out (14), suggesting that SFs have anabolic effects on joint homeostasis and are involved in the restoration process of articular cartilage. We demonstrated that adenovirus vector–mediated ALK3^{CA} gene expression induced robust induction of chondrocyte-specific gene expression in SFs in a ligand-independent manner. Clear induction of Sox9, a key transcription factor regulating chondrogenesis (36, 37), followed by type II collagen and aggrecan expression, was observed in the ALK3^{CA}-expressing cultures, while type X collagen was only weakly induced in the cultures and no osteocalcin expression could be found (Figures 2 and 5). Induction of these chondrocyte-specific genes through ALK3^{CA} expression was not observed in skin fibroblasts, suggesting the cell specificity of the events (data not

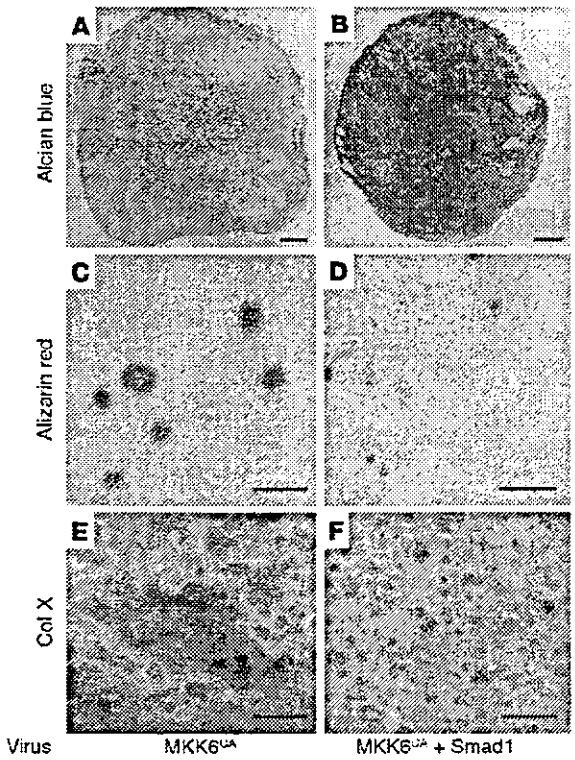

Figure 6
Induction of Alizarin red staining and type X collagen in MKK6-transduced SFs in pellet cultures. (**A–F**) SFs infected with MKK6^{CA} virus alone (**A**, **C**, and **E**) or together with Smad1 virus (**B**, **D**, and **F**) were subjected to pellet culture. Cultures were fixed with 3.7% formaldehyde 3 weeks later, and then stained with Alcian blue (**A** and **B**), Alizarin red (**C** and **D**) or anti–type X collagen (**E** and **F**). Note the increased Alcian blue staining and the reduced Alizarin red activity and type X collagen immunoactivity with Smad1 coexpression. Scale bars: 100 μm (**A–D**) and 50 μm (**E** and **F**).

shown). The chondrogenic effect of ALK3^{CA} virus was further confirmed histologically by pellet cultures performed in vitro and in vivo (Figures 3 and 4). Induction of neither the osteoblast markers osteopontin and osteocalcin nor the terminal chondrocyte differentiation markers type X collagen and mineralization was observed in ALK3^{CA}-expressing cells (Figures 2, 3, 5, and 6). These results suggest that ALK3 signaling, that is, BMP signaling, has both stimulatory and regulatory roles in chondrogenesis: to induce the chondrogenic differentiation of SFs and at the same time to block their osteoblastic or hypertrophic differentiation. Despite the structural similarity between ALK3 and ALK6, the ALK6^{CA} virus was much less efficient in chondrogenesis, the reason for which remains to be clarified. Although many studies have demonstrated a prochondrogenic effect for TGF-β (15, 16, 20–22), we failed to find an anabolic effect for ALK5^{CA} which is expected to mimic TGF-β signaling, on the chondrogenic differentiation of SFs. We cannot fully explain the discrepancy between our results and those of previous studies, but Robbins and coworkers recently reported that adenovirus vector–mediated TGF-β gene transduction into arthritic joints in fact exacerbated cartilage degradation (38), raising the possibility that sustained activation of TGF-β signaling, via ALK5, has instead a negative effect on chondrogenesis. Further study will be required to elucidate the difference between TGF-β and BMP signaling.

The signaling of TGF-β/BMPs is transduced by Smad family members (31, 34, 35). Receptor-regulated Smads (R-Smads) are direct substrates of type I receptors and are phosphorylated at the C-terminal SSV/MS motif. R-Smads then form heteromeric complexes with common-mediator Smads and translocate into the nuclei, where they regulate transcription of target genes. In addition to Smad pathways, there is evidence that MAP kinase cascades are also implicated in ALK signaling, in which TGF-β–activating kinase (TAK1), a member of the MAP kinase kinase kinase family, plays a key role. TAK1 activates MAP kinase kinase in combination with an adaptor molecule, TAB1, which leads to JNK and p38 activation (32). The role of p38 in chondrogenesis has recently attracted particular interest because p38 inhibitors such as SB203580 suppress the chondrogenic differentiation of ATDC5 cells induced by growth/differentiation factor-5 (33, 39). However, the exact roles of the Smad pathways and p38 pathways in chondrocyte differentiation are not yet fully clarified. We used a combination of adenoviral gene delivery and a chemical inhibitor to segregate the roles of these two pathways downstream of ALK3 activation and found that (a) inhibitory Smad (Smad6) expression or treatment with the p38 inhibitor SB203580 suppressed the effect of ALK3^{CA} expression (Figure 2) (b) Smad1 synergistically augmented the effect of ALK3^{CA} (Figure 5A), and (c) activation of p38 pathways alone by MKK6^{CA} expression induced the hypertrophic differentiation markers type X collagen and mineralization in SFs, which was suppressed by Smad1 coexpression (Figures 5B and 6). These results suggest that although both Smad and p38 activation is necessary for chondrogenic differentiation of SFs, sustained activation of p38 pathways alone prompts the terminal differentiation of the cells. Consistent with our results, Zhen et al. (40) reported that parathyroid hormone inhibits type X collagen expression in hypertrophic chondrocytes by suppressing p38 pathways. Von der Mark et al. (41) reported the focal appearance of type X collagen in osteoarthritic cartilage, which may be involved in the degenerative changes of the articular cartilage and in the pathogenesis of osteoarthritis. Using the mouse model of osteoarthritis, we found that activated p38 is associated with type X colla-

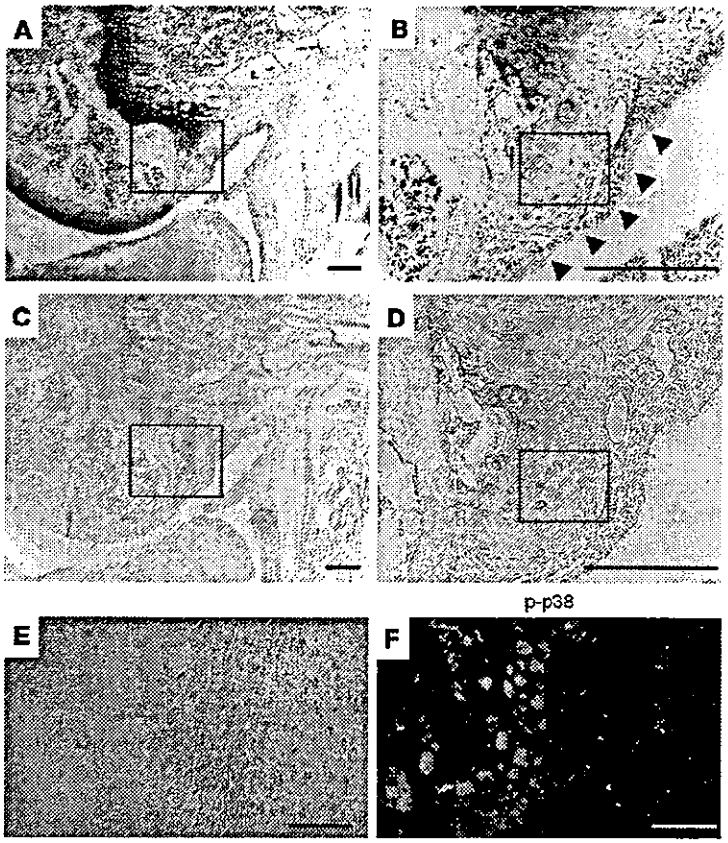

Figure 7
Histological analysis of knee joints in the mouse ACL and MM resection model. (**A–F**) Toluidine blue staining (**A** and **B**) and type X collagen immunostaining (**C** and **D**) at the marginal area between the articular cartilage and synovium. **B** and **D** present higher-magnification views of **A** and **C**, respectively. Osteochondrophytes were formed at the posterior edge of the femoral condyle, and they were positively stained by anti–type X collagen as well as toluidine blue (rectangular areas in **A** and **C**). Clusters of migrating synovial cells were observed adjacent to the osteochondrophytes (**B**, arrowheads) where future osteochondrophytes will develop, and they were positively stained by anti–type X collagen at the marginal area between synovium and osteophytes (rectangular area in **D**). This region was also positively stained by anti–phospho-p38 (**F**). **E** and **F** represent phase-contrast microscopy (**E**) and immunostaining with anti–phospho-p38 (**F**) of the rectangular area in **D**. Positive phospho-p38 staining was observed at the area of osteochondrophytes as well as the marginal synovium. Scale bars: 500 μm (**A–D**) and 50 μm (**E** and **F**).

gen expression in the synovial tissues adjacent to osteochondrophytes as well as in the degenerative cartilage (Figure 7).

Smad pathways not only are required for chondrogenic differentiation of SFs but also critically regulate the stage of differentiation of the cells and suppress their terminal differentiation process. Consistent with our findings, Scharstuhl recently reported inhibitory action of Smad7 in TGF-β-induced chondrocyte proliferation and proteoglycan production (42), indicating a critical role for Smad pathways. Hidaka and coworkers (43) demonstrated that adenovirus vector–mediated BMP-7 expression in chondrocytes accelerates the cartilage repair process. More recently, Lories and colleagues (44) demonstrated that BMP-2 and BMP-6 expressed in arthritic synovium are regulated by proinflammatory cytokines and differentially modulate fibroblast-like synoviocyte apoptosis, and Fukui et al. (45) found that BMP-2 expression was increased by proinflammatory cytokines in normal and osteoarthritis chondrocytes. These findings, combined with our observations, suggest that although BMPs have favorable effects on the repair process of articular cartilage, they may have proapoptotic and/or degenerative effects on the cells when p38 pathways are overactivated. Our findings suggest an important role for p38 signal transduction pathways in chondrocytes and SFs, leading to degenerative joint disorders, and suggest the potential utility of p38 modifiers in the treatment of rheumatoid arthritis and/or osteoarthritis. In fact, p38 kinase modifiers are now in clinical trials to treat rheumatoid arthritis (46). Based on our observations, we would like to propose that SFs are an excellent source for chondroprogenitors, which can be differentiated into chondrocytes via ALK3 activation, and that activation of the Smad pathway while controlling the degree of p38 activation may be a way to generate committed chondrocytes for the repair and/or replacement of cartilage.

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REVIEW ARTICLE

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Intracellular signal transduction pathways: good therapeutic targets for joint destruction in rheumatoid arthritis

Abstract Preventing joint destruction is one of the most challenging issues in treating patients with rheumatoid arthritis (RA), and I propose that intracellular signaling pathways in osteoclasts and synovial fibroblastic cells (SFCs) can be good therapeutic targets. Osteoclasts are primarily involved in the bone destruction in RA joints, and SFCs support osteoclast differentiation and activation by producing various proinflammatory cytokines including receptor activator of NF-κB ligand (RANKL), the osteoclast differentiation factor belonging to the tumor necrosis factor-α superfamily. Suppressing c-Src pathways by adenovirus vector-mediated C-terminal Src family kinase (Csk) gene or Ras/extracellular-regulating kinase (ERK) pathways by introducing dominant negative Ras (Ras^{DN}) adenovirus reduced osteoclastic bone resorption as well as the abnormal proliferation and interleukin-6 production of SFCs, and the local injection of these viruses ameliorated the joint destruction in adjuvant arthritis rats. Moreover, chondrogenic differentiation of SFCs could be induced by stimulating activin receptor-like kinase 3 pathways.

Key words Adenovirus · Osteoclast · Rheumatoid arthritis (RA) · Synovial fibroblast cells (SFCs)

Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disorder with an unknown etiology characterized by the invasive synovial hyperplasia leading to the progressive joint destruction.[1] Radiographic studies have shown that the bone erosion in RA begins at the early stage of the disease, and gradually exacerbates. Bone erosion results in the severe deformity of the affected joints and impairs the normal activity and the quality of life of the RA patients, and hereby, preventing such devastating states is one of the most challenging issues in treating them. Because the exact etiology and pathology of RA remains unknown, most treatments of RA just treat symptoms of the disease. Non-steroidal anti-inflammatory drugs, including cyclooxygenase 2 inhibitors, have been prescribed to reduce the painful symptoms of the disease, but they have little effect on stopping the progression of the joint destruction. Recent studies have revealed that some disease-modifying antirheumatic drugs and biological agents such as anti-tumor necrosis factor (TNF)-α antibody ameliorate the progression of the joint destruction in RA.[2] However, the bone-protective effect of these reagents is limited in most cases, and their long-term effects have not been established yet. Moreover, the prolonged usage of these medicines sometimes causes severe side effects. Therefore, novel therapeutic interventions specifically targeting the joint destruction in RA are greatly expected.

Proliferating synovium produces an elevated amount of proinflammatory cytokines interleukin (IL)-1, IL-6, IL-17, and TNF-α, and matrix-degenerating enzymes matrix metalloproteinases and cathepsins, which are involved in the bone and cartilage destruction.[1,3] Considerable data have demonstrated that synovial fibroblastic cells (SFCs), type B synovial cells with fibroblastic morphology, are one of the principal cells implicated in the arthritic conditions in RA.[4] In RA, SFCs markedly increase in number and display transformed phenotypes, and the activation of various protooncogenes including *myc*, *ras*, and *fos* is involved in the abnormal growth rate and transcriptional activity of the cells.[5] Bone erosion usually begins at the interface of the cartilage and the proliferating synovium, and bone-resorbing osteoclasts can be observed at the erosive synovium/bone interfaces in RA joints. Accumulating evidence has revealed that osteoclasts, primary cells responsible for bone resorption, are involved in the bone destruction in RA, and recent progress in the molecular biology and biochemistry has elucidated the molecular mechanism of the osteoclast differentiation and activation. In contrast to such catabolic

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